Cortisol Levels as a Determinant of Disease and Health Status in Animals

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Abstract: The veterinarian is daily faced with the challenge of diagnosing several disease conditions in different animals. Measurement of serum cortisol concentration has been used in the assessment of stress and pain caused by infectious and non-infectious disease conditions, mismanagement, transportation, adverse environmental temperature and surgical operations. The hormone-cortisol, is involved in numerous metabolic and immunologic functions and can thus serve as a marker to monitor animal welfare. Several factors affect the specific test to be carried out and which sample is to be used. Recently, RIA has been identified as the gold standard for determination of cortisol levels. However it also has its own short comings hence the need to utilize other assay techniques such as ELISA, fluorescence techniques and chemical assays which are more common in our environment. There is also prospect for the analysis of cortisol using non-invasive samples such finger nails as well as ear wax for enhanced test results.

Introduction

Cortisol (hydrocortisone; 11, l7, 21-trihydroxy-pregnene-3, 20- dione) is a 21-carbon steroid secreted by the adrenal cortices, and is the most physiologically active of the naturally occurring glucocorticoids (Ali *et al.,* 2011). In farm animals, measurement of serum cortisol concentration has been used in the assessment of stress and pain caused by mismanagement, travel, inappropriate environmental temperature, castration without local anesthesia, and disease (Raff *et al.,* 2012). Physiological actions of cortisol include regulation of carbohydrate metabolism, electrolyte balance, water distribution and immunosuppressant and anti-inflammatory activity. Cortisol is involved in numerous metabolic and immunologic functions (Keevil, 2016). Measurement of serum cortisol concentration is common in large animal medicine to monitor effects of modern farming practices on animal welfare (Raff *et al.,* 2012).

Synthesis and Regulation of Cortisol

Cortisol is a glucocorticoid produced by the adrenal cortex in response to adrenocorticotropic hormone (ACTH). Cortisol binds to two intracellular receptors, the mineralocorticoid receptor (MR), and the glucocorticoid receptor (GR) (Yoon *et al*., 2015). Of the two receptors, the MR has the higher affinity for cortisol. This receptor will be almost completely occupied by cortisol at levels too low to activate the GR. 311β-Hydroxysteroid dehydrogenase (Type 2; 11β-HSD2) converts cortisol to inactive cortisone (Gold *et al.,* 2006). This enzyme is expressed predominantly in mineralocorticoid target tissues including kidney, colon, and salivary gland where it serves to protect the MR from glucocorticoid excess. Individuals lacking this enzyme exhibit a syndrome known as apparent mineralocorticoid excess which features hypertension and hypokalemia. The enzyme 11β-HSD1 is a key regulator of intracellular glucocorticoid levels, catalyzing the regeneration of cortisol from cortisone (Friedman *et al.,* 1995). Visceral adipose tissue from obese humans has increased 11β-HSD1 activity compared to adipose tissue obtained from normal individuals. Cortisol strongly promotes adipocyte differentiation; mature visceral adipocytes express high levels of the glucocorticoid receptor. Cortisol circulates largely in protein-bound forms, the majority being attached to corticosteroid binding globulin (Cirimele *et al* 2000). The half-life of cortisol in the circulation is about 80 minutes, with approximately 1% excreted unchanged in the urine. This excreted fraction is called urinary "free cortisol" and if renal function is normal, will reflect the level of circulating non-protein bound

cortisol. After metabolic breakdown, mainly in the liver, cortisol is excreted into the urine as dihydro and tetrahydro-derivatives conjugated to glucuronic acid (Friedman *et al.,* 1995).

Factors Affecting Cortisol Concentration

The circulating cortisol concentration is normally subject to a circadian rhythm, with the maximum level being reached at 8-9 a.m. and the minimum around midnight. Concentrations are usually elevated in pregnancy and in patients receiving high dose estrogen therapy (Friedman *et al.,* 1995). Anomalous cortisol concentrations can result from stimuli such as trauma, fear, fever, shock, hypoglycemia and depression. In pathological states of the HPA axis, elevated or depressed values of cortisol may be found (Loussouarn *et al.,* 2001). Adrenal tumors and pituitary or ectopic adrenocorticotrophic hormone (ACTH)-producing tumors are frequently associated with cortisol over-production (Cushing's syndrome), while adrenal insufficiency results in cortisol under-production (Addison's disease) (Gold *et al.,* 2006). The production of glucocorticoids is increased by stress, therefore, cortisol can be used as a biomarker of stress. Cortisol levels increase with age, and are often elevated in major depressive disorder, certain forms of hypertension, and AIDS. Serum cortisol concentration also varies due to, diet, environmental temperature, or humidity and physiological conditions. Pharmacological treatment with glucocorticoids can result in cognitive impairment, decreased bone density, hypertension, and an increased risk of development of type II diabetes (Yoon *et al.,* 2015).

Methods of Measuring Cortisol Levels

Cortisol can be measured in many matrices including blood, feces, urine, hair and saliva. Serum cortisol concentrations range from about 25-800 nM (9-300 ng/ml) and approximately 90-95% of the cortisol is bound to proteins. Urinary cortisol is not bound to proteins, but its levels are dependent on glomerular and tubular function. In saliva, approximately 67% of cortisol is unbound. There is generally good correlation between cortisol measurements in saliva and serum (Friedman *et al.,* 1995).

Hair Cortisol Analysis Using ELISA Technique

This is a unique type of enzyme linked immunesorbent assay. A minimum of 10 mg of hair from the scalp end was used for each sample. The hair is weighed, cut into small pieces using small surgical scissors, put into a disposable glass scintillation vial and 1 ml of methanol was added. The scintillation vial is sealed and incubated overnight (~16 hr) at 52 °C while shaking (Gyromax® Amerex Instruments Inc.) (Ito *et al.,* 2005). After incubation, the supernatant was removed and put into disposable glass culture tubes. The supernatant is evaporated in a dry bath (Thermolyne® Dri-Bath) under nitrogen (Techne® Sample Concentrator) until completely dry. Once the methanol is removed, the sample is resuspended in 150-250 µL of phosphate buffered saline (PBS) at pH 8.0. Samples are vortexed for one minute followed by another 30 seconds until they are well mixed (Loussouarn *et al.,* 2001). The cortisol in the hair samples was measured using the Salivary ELISA Cortisol kit© (Alpco Diagnostics®, Windham, NH) as per the manufacturer's directions with the reagents provided. Basically, an enzyme catalyses the hydrolysis of substrates that produce a coloured product. The extent of colour change is used to quantitate the amount of labeled hormone-antibody complex that is present (Layker *et al.,* 2003).

Thus, the reference range for cortisol levels in hair of healthy non-obese individuals is 17.7-153.2 pg/mg of hair with a median of 46.1 pg/mg (Ito *et al.,* 2005).

Advantages.

Its collection is non-invasive and can be performed by non-health care workers at any time of the day. Samples can be stored at room temperature and be sent by mail, making it potentially useful in population studies. Further, levels reflect average hormone levels over the last two months, as opposed to blood, saliva and urine samples, which reflect acute or daily cortisol levels. Another important characteristic of measurement of cortisol in hair is that the levels are not affected by acute stress. Hair cortisol may be important in the diagnosis of cyclical Cushing's syndrome, and in depression, in which daily cortisol excretion in urine is increased in a higher number of days, but not all days (Ito *et al.,* 2005).

Limitations.

It is restricted to individuals who have sufficient hair at the posterior vertex and do not have cultural/religious objections to taking a hair sample. Hair cortisol levels are not able to determine brief cortisol responses, and cannot be used to determine day-to-day variation. It is not known whether hair cortisol levels vary with hair growth rate, which could be important as the activity of hair follicles is intermittent, consisting of active phase (anagen), transitional (catagen) and resting phase (telogen) (Loussouarn *et al.,* 2001). Further, hair growth rate decreases with age, in various diseases (e.g. hyper- and hypothyroidism) and varies among ethnic groups. For saliva samples the saliva flow rate has only a minor effect on saliva cortisol levels. By analogy, it is conceivable that for hair cortisol levels the hair growth rate may have limited importance. Each kit also has a different specification. Finally, levels of cortisol in hair may be affected by local synthesis or

metabolism in the hair follicles, although the relative contribution of locally produced hormones may be limited. Currently it is not known if hair levels of cortisol vary throughout the various seasons, or if hair growth rate follows a diurnal rhythm (Layker *et al.,* 2003).

Liquid Chromatography

In this chemical assay, purification of cortisol is achieved using open column chromatography, but high performance liquid chromatography is more commonly used as an analytical technique. HPLC uses high-resolution columns containing very uniform particles. These columns can separate very similar molecules with high efficiency, and normally operate under high pressure (Keevil *et al*., 2016).

Mass spectrometry

Mass spectrometry can be used to identify the molecular weight, structure and position of functional groups of small molecules. The mass spectrometer produces charged ions from the sample, consisting of parent ion and fragments of original molecule, and then sorts these ions by mass/charge ratio in a magnetic field (Jones *et al.,* 2012). The relative numbers and the charge to mass ratio of each ion is characteristic of a particular compound and can be used to identify the structure of the compound. It can also be linked to a gas chromatography or liquid chromatography to identify compounds such as cortisol that is separated by chromatography (Keevil *et al*., 2016).

Radioimmunoassay Technique (RIA KIT)

Radioimmunoassay (RIA) is the "gold standard" method for evaluation of serum cortisol concentration (Deboro *et al.,* 2016). This assay is based on the competition between unlabelled cortisol and fixed quantity of 125I-labelled cortisol for limited number of binding sites on cortisol specific antibody. Allowing to react a fixed amount of tracer and antibody with different amounts of unlabelled ligand, the amount of tracer bound by the antibody will be inversely proportional to the concentration of unlabelled ligand. During a 2-hour incubation period with continuous agitation, immuno-complex is immobilized on the reactive surface of test tubes. After incubation the reaction mixture is discarded, and the radioactivity is measured in a gamma counter (Dimitris *et al.,* 2012). The concentration of antigen is inversely proportional to the radioactivity measured in test tubes. By plotting binding values against a series of calibrators containing known amounts of cortisol, a calibration curve is constructed, from which the unknown concentration of cortisol in patient samples can be determined Deboro *et al.,* 2016).

Methodology

1. Equilibrate reagents and samples to room temperature before use (min. for an hour).

2. Label coated tubes in duplicate for each standard (S1-S6), control serum (C) and samples (Sx). Optionally, label two uncoated test tubes for total count (T).

3. Homogenize all reagents and samples by gentle mixing to avoid foaming.

4. Pipette 10 μl each of standards, control and samples into the properly labelled tubes.

5. Pipette 500 μl of tracer into each tube.

6. Pipette 500 μl antiserum into each tube except T.

7. Fix the test tube rack firmly onto the shaker plate. Seal all tubes with a plastic foil. Turn on the shaker and adjust an adequate speed such that liquid is constantly rotating or shaking in each tube.

8. Incubate tubes for 2 hours at room temperature.

9. Aspirate or decant the supernatant from all tubes by the inversion of the rack. In the upside down position place the rack on an absorbent paper for 2 minutes.

10. Count each tube for at least 60 seconds in a gamma counter.

11. Calculate the cortisol concentrations of the samples as described in calculation of results.

Sensitivity: 2, 9 nmol/l, defined as the concentration, 2 standard deviations from the zero standard (Deboro *et al.,* 2016).

Advantages

It is simple, fast, inexpensive and can be automated resulting in a very fast turn-around time (Perogamyros *et al.,* 2012).

Limitations

The reagents supplied in this kit are optimized to measure cortisol levels in serum and plasma. Avoid freezing and thawing of reagents and specimens. Hemolyzed and lipemic specimens may give false values and should be avoided. Components from various lots or from kits of different manufacturers should not be mixed or interchanged (Perogamyros *et al.,* 2012).

Short shelf-lives of the radioactive reagents, risk of radiation exposure for staff, and the need to dispose of toxic waste (Deboro *et al.,* 2016).

Acetylcholinesterase Competitive ELISA Technique

Cortisol ELISA Kit can be used for quantification of cortisol in urine, plasma, and other sample matrices. The assay has a range from 6.6-4,000 pg/ml and a sensitivity (80% B/B$_0$) of approximately 35 pg/ml (Gallagher *et al*., 2006).

This assay is based on the competition between cortisol and cortisolacetylcholinesterase (AChE) conjugate (cortisol tracer) for a limited number of cortisol-specific mouse monoclonal antibody binding sites. Because the concentration of the cortisol tracer

is held constant while the concentration of cortisol varies, the amount of cortisol tracer that is able to bind to the Cortisol mono-clonal antibody will be inversely proportional to the concentration of cortisol in the well. This antibody-cortisol (either free or tracer) complex binds to the goat polyclonal anti-mouse IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's Reagent (which contains the substrate to AChE) is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm (Glass et al., 1984). The intensity of this color, determined spectrophotometrically, is proportional to the amount of cortisol tracer bound to the well, which is inversely proportional to the amount of free cortisol present in the well during the incubation; or Absorbance $\propto$ (Bound Cortisol Tracer) $\propto$ 1/(Cortisol; Gallagher *et al*., 2006).

Methodology

Addition of the Reagents

1. ELISA Buffer

Add 100 μl ELISA Buffer to NSB wells. Add 50 μl ELISA Buffer to B_0 wells. If culture medium was used to dilute the standard curve, substitute 50 μl of culture medium for ELISA Buffer in the NSB and B_0 wells (i.e., add 50 μl culture medium to NSB and B_0 wells and 50 μl ELISA Buffer to NSB wells).

2. Cortisol ELISA Standard

Add 50 μl from tube #8 to both of the lowest standard wells (S8). Add 50 μl from tube #7 to each of the next two standard wells (S7). Continue with this procedure until all the standards are aliquoted. The same pipette tip should be used to aliquot all the standards. Before pipetting each standard, be sure to equilibrate the pipette tip in that standard.

3. Samples

Add 50 μl of sample per well. Each sample should be assayed at a minimum of two dilutions. Each dilution should be assayed in duplicate (triplicate recommended).

4. Cortisol AChE Tracer

Add 50 μl to each well except the TA and the Blk wells.

5. Cortisol ELISA Monoclonal Antibody

Add 50 μl to each well except the TA, the NSB, and the Blk wells.

Incubation of the Plate

Cover each plate with plastic film (Item No. 400012) and incubate overnight at 4°C.

Development of the Plate

1. Reconstitute Ellman's Reagent immediately before use (20 ml of reagent is sufficient to develop 100 wells): 100 dtn vial Ellman's Reagent (96-well kit; Item No. 400050): Reconstitute with 20 ml of UltraPure water

2. Empty the wells and rinse five times with Wash Buffer.

3. Add 200 μl of Ellman's Reagent to each well.

4. Add 5 μl of tracer to the TA wells.

5. Cover the plate with plastic film. Optimum development is obtained by using an orbital shaker equipped with a large, flat cover to allow the plate (s) to develop in the dark. This assay typically develops i.e., B_0 wells ≥0.3 A.U. (blank subtracted) in 90-120 minutes.

Reading the Plate

1. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, etc.

2. Remove the plate cover being careful to keep Ellman's reagent from splashing on the cover.

NOTE: Any loss of Ellman's reagent will affect the absorbance readings. If Ellman's reagent is present on the cover, use a pipette to transfer the Ellman's reagent into the well. If too much Ellman's reagent has splashed on the cover to easily redistribute back into the wells, wash the plate three times with Wash Buffer and repeat the development with fresh Ellman's reagent.

3. Read the plate at a wavelength between 405 and 420 nm. The absorbance may be checked periodically until the B0 wells have reached a minimum of 0.3 A.U. (blank subtracted). The plate should be read when the absorbance of the B_0 wells are in the range of 0.3-1.5 A.U. (blank subtracted; Tanada *et al.,* 1998).

The disadvantages of this test is that it is time-consuming, the cost of the kit, risk of contamination via staff, and need for staff in every step of the method (Gallagher *et al*., 2006).

Enzyme-Linked Fluorescent Assay (VIDAS Assay Technique)

The system uses a fluorescent label such as fluorescein isothiocyanate, which has an excitation maximum at 485nm and emission maximum at 525nm. The VIDAS cortisol test is an enzyme-linked fluorescent assay designed for the Mini Vidas system. Immediately after thawing of serum sample, cortisol is measured following the manufacturers instruction on the Mini Vidas analyzer (BioMerieux S.A., Lyon, France; Tunn *et al.,* 1992). According to the manufacturer the assay has a measurement range of 5.51–2759 nmol/L for human serum. The analyzers were cleaned, calibrated, and operated in accordance with the manufacturer's instructions. The Mini-Vidas analyzer is simple and fast to operate. The Mini Vidas is a compact, rapid, automated immunoassay analyzer that needs to be calibrated only once every 14 days, optimizing the per result cost. Tests sharing similar protocols may be run together in 1 section of the analyzer and each section functions independently from the other. Moreover, it is possible to perform a

single test with single dose reagents (Tunn *et al.,* 1992). The rapidity of measurement (30 minutes), small sample required (200L), and wide working range make the VIDAS method suitable for field studies in farm animals. This technique is more relevant when large numbers of animals, in particular where the mean value of the group is relevant—such as studies on stress. On the other hand, when greater accuracy and precision are needed for clinical assessment of the individual subject, this study suggests that this method is not advisable due to its relatively low specificity (76%) (Tanada *et al.,* 1998).

Fluorometric Measurement of Urinary Free Hydrocortisone (Cortisol)

Free steroids are extracted from urine with di-chioromethane and washed with aqueous alkali to remove phenolic steroids. Corticosteroids in the washed extracts are then treated with acid-alcohol for fluorogenesis. The il-hydroxy and C-3 ketone (ring A) are primarily responsible for fluorescence, whereas the 17-hydroxy group exerts little influence on fluorescence. Fluorometric determination of corticosteroids, then, has resulted in the generic term "11-hydroxycorticosteroid" (11-OHCS) to differentiate such methods from those based on the Porter-Silber reaction (17-OHCS) (Tunn *et al.,* 1992).

Procedure

1. Prepare unknown, standard, and blank by adding 2.0 ml of either urine, working cortisol standard, or water, respectively, to labeled 50-mi glass-stoppered centrifuge tubes.

2. Add 15.0 ml of dichloromethane to all tubes, stopper, and shake thoroughly. Complete the extraction by mixing each tube on a vortex-type mixer for 1mm.

3. Allow the phases to separate, then remove and discard the aqueous (upper) layer.

4. Add 2.0 ml of sodium hydroxide (0.1mol/liter) to all tubes, and mix (vortex) for 1mm. Remove and discard the alkaline (upper) layer.

5. Wash residual alkali from the extracts by adding 2.5 ml water, and mixing the contents of the tubes for 30s (vortex); then remove and discard the aqueous (upper) layer.

6. At zero time (set a timer for 15 mm) add 10.0 ml of the washed dichlormethane extracts to 5.0 ml of acid-alcoholreagent (warmed to room temperature) in labeled tubes, stopper, and mix for 30s on the vortex mixer.

7. Immediately remove the dichloromethane (upper) layer from each tube, and discard.

8. Transfer the acid-alcohol phases to square quartz cuvets (1-cm lightpath), and measure the fluorescent intensity exactly 15 mm from zero time. A small proportion of circulating blood cortisol is excreted in the urine in the unconjugated or free-state

and consequently this fraction increases or decreases in accordance with adrenal output. For this reason, fluorometric determination of urinary free cortisol is most informative in detecting hypergiucocorticoidism, and has some advantages over colorimetric measurements of 17-hydroxy-corticosteroids (17-OHCS) and 17-ketogenic steroids (17-KGS). In addition to its use in assessing hypercortisolism, we have used the fluorometric assay described here to detect hypocortisolism. In Addison's disease and panhypopituitarism, values are found that are clearly lower than those of "normal" subjects. Drugs such as spironolactone result in marked fluorescence, thus altering the results. They should not be used prior to the test (Umeda *et al.,* 2015).

Electrochemiluminescence Measurement of Salivary Cortisol

An electrochemiluminescence (ECL) method using a routine automated immunoassay analyser was introduced to measure SC levels (Raff *et al.,* 2012). It has been reported that it provides superior analytical performance even in very low concentration of analyte and a shorter turnaround time (Perogamyros *et al.,* 2012). This method seems to be faster and as convenient. Saliva samples are collected using oral swabs (Salimetrics oral swab; Salimetrics Europe Ltd., Suffolk, United Kingdom) made of a non-toxic, inert polymer shaped into a 30x10 mm cylinder leading to a passive droll of saliva (Raff *et al.,* 2012). The oral swabs are placed under the front of the tongue and waited for 1 or 2 minutes until they are saturated completely (Perogamyros *et al.,* 2012). After removal of the moisturised swab, it was kept in a swab storage tube measuring 17x100 mm (Salimetrics saliva storage tube; Salimetrics Europe Ltd., Suffolk, United Kingdom), which consists of a capped, conical polypropylene centrifuge tube with a separate insert that allows saliva to be centrifuged into the bottom of the conical tube (Yoon *et al*., 2015). When they were still at room temperature, the tubes were vortexed, and then were immediately centrifuged for 15 minutes at approximately 3.000 RPM. After centrifugation, the swab and small insert were thrown away, and the large outer tube was stored at -80 °C until analysis (Perogamyros *et al.,* 2012). On the day of measurement, the samples were brought to room temperature. Assays should be performed using only clear saliva, avoiding any sediment present in the bottom of the tube. SC levels is measured by using an Elecsys Cortisol assay in the same way as for serum or plasma specimens. Two hundred mililitres of salivary samples were transferred to an Elecsys sample cup and measured by a "cobas e 601" an analyser (Roche Diagnostics GmbH; Mannheim, Germany) (Raff *et al.,* 2012).

Determination of Cortisol by Radiostereoassay or Competitive Protein Binding

Corticosteroid binding globulin (CBG) was prepared from plasma sample. The plasma was stripped of steroids with charcoal Norit A in a concentration of 50 mg/ml of plasma, filtered twice through Whatman NQ 50 filter paper (Fisher Scientific Co.) and stored in 1 ml aliquots at -10 ^{0}C. The scintillation fluid was composed of 42 ml of liquofluor (New England Nuclear Co.), 21 mI of absolute ethanol and 6.5 ml of Biosolv-solubilizer (Beckman NQ 184983) in 1 liter of toluene (spectroanalysed grade) (Umeda et al., 2015).

Extraction: A tracer solution of 3H-cortisol 500-600 cpm in 0.1 ml of ethanol was placed in 15 ml stoppered centrifuge tubes and dried. Unknown plasma in amounts of 0.2-0.5 ml was added. Extraction of this was performed with two 5 ml aliquots of methylene chloride and each tube was shaken in a vortex mixer for 1 minute. The methylene chloride was evaporated under nitrogen, and the sample was reconcentrated by dissolving in 2 ml of acetone and redrying (this last procedure increased the recovery considerably) (Perogamyros et al., 2012).

Thin Layer Chromatography (TLC):

The separation of the steroids was performed by TLC using fluorescent silica gel. The system used to develop the TLC was methylene chloride ethanol 92:8. Cortisone and corticosterone interfere with cortisol in the binding sites of corticosteroid binding globulin. Solutions of these steroids run faster than cortisol in this system and so are easily separated. The speed of the spots can be controlled with the concentration of the alcohol. Two and a half cm of the 2 cm wide silica gel stripes were scraped off the plates and diluted with 7 ml of acetone through disposable pipettes containing acetone washed glass wool. The diluent was collected in 5 ml centrifuge tubes in which the complete binding procedure was performed. The sample was diluted in 2 ml of acetone and a 0.5 ml aliquot was placed in a counting vial to detennine the percentage of recovery (Perogamyros et al., 2012).

Binding Procedure:

On a standard curve, each set of determinations carried at least four standard replicates and three O, 10, 20, 40 and 80 ngs replicates. The corticosteroid binding globulin solutions were prepared by diluting the stripped plasma to a 1.5%. Enough 3H-cortisol was added to give a final concentration of 10 ng% of the solution. One ml of the CBG-isotope solution was added to each tube of the standard curve and of the unknown samples, and these were shaken on a vortex mixer. The samples were incubated at 45 e for 5 min and then cooled at 8 ^{0}C for 10 min. Fifteen mg of fuller's earth were added and each was shaken briefly on a vortex mixer, and a further 2 min on a rotating agitator. The tubes are then cooled at 8 C for another 10 min. After centrifugation for 2 min at 2500 rpm, 0.5 ml of the supernatant was placed in a counting vial. Ten ml of scintillation fluid is added to the vial and counted in a Liguid Scintillation Counter (Nuclear Chicago Mod. 720) with an efficiency of 15% (Yoon et al., 2015).

Modified assay of Porter-Silber Chromogens in Urine

Acidify an aliquot of filtered urine (from a 24-hr. collection) to pH I in a 100-ml beaker with 50% sulfuric acid using a PH meter. Saturate 5 ml. of the acidified sample with ammonium sulfate in a 50-mi.screwcap centrifuge tube. Add crystals with shaking until only traces remain.

At the bottom. Add 30 ml. of cold ethyl ether, cap the tubes, and invert twice, release mg the pressure each time by loosening the cap slightly. Extract by vigorous shaking for 60 sec. (Yoon et al., 2015). Allow the layers to separate. Centrifuge, if necessary, at 1500 rpm for 1mm. Aspirate off the aqueous (bottom) layer by means of a blunt-tip needle. Filter into a marked tube through Whatman No. 1 filter paper. Evaporate the ether extract in a hood or under nitrogen. Dissolve the ether residue in 5ml. of methanol. Should any precipitate appear, centrifuge and obtain clear supernatant for color development. The prepared reagent is stored in an amber bottle and kept refrigerated at 40^0 and Used for 17-OHCS determination after ACTH stimulation (Perogamyros et al., 2012).

Colorimetry

Prepare and mark tubes or cuvets divided into 2 groups. Add sulfuric acid reagent to each Group. Thoroughly mix by vigorous shaking (parafilm capping) or stirring by mechanical mixer for 15 sec. Incubate in a 56° (176) serological bath for 20 mins. At the end of the period, place the tubes in an ice bath or cold- water trough for 3 mins. Read the absorbance (0.1) at 410 nl against reagent blank (RB; Yoon et al., 2015).

Conclusion

Determination of cortisol levels is essential in determining health and disease states. Several factors affect the specific test to be carried out and which sample is to be used. Recently, RIA has been identified as the gold standard for determination of cortisol levels. However it also has its own short comings hence the need to utilize other assay techniques such as ELISA, fluorescence techniques and chemical assays which are more common in our environment. There is also prospect for the analysis of cortisol using non-invasive samples such finger nails as well as ear wax for enhanced test results.

References

1. Achenbach, T.M., Edelbrock, C.S., (1984). Psychopathology of childhood. *Annual Review of Psychology*. 35, 227e 256.
2. Adam, E. K., Till, H. L., Granger, D.A., (2011). Diurnal alpha amylase patterns in adolescents: associations with puberty and momentary mood states. *Biology of Psychology*. 88, 170e 173.
3. Ali, N., Pruessner, J.C., (2012). The salivary alpha amylase over cortisol ratio as a marker to assess dysregulations of the stress systems. *Physiology of Behaviour*. 106, 65e72.
4. Cirimele, V, Kintz, P, Dumestre, V, Goulle, JP, Ludes, B. Identification of ten corticosteroids in human hair by liquid chromatography-ionspray mass spectrometry. Forensic Science International. 2000; 107: 381-8.
5. Debono, M., Harrison, R. F, Whitaker, M. J., Eckland, D., Arlt, W., Keevil, B. G., Ross, R. J. (2016). Salivary cortisone reflects cortisol exposure under physiological conditions, Science, 3; 34-45.
6. Dimitris, A., Papanicolaou, J. A. Yanovski, G. B. Cutler, J. R. George P. Chrousos, J. (2012). A Single Midnight Serum Cortisol Measurement Distinguishes Cushing's Syndrome from Pseudo-Cushing States. *Journal of Clinical Endocrinology and Metabolism.32, 443-444*.
7. Friedman, T. C, Yanovski, J.A.1995 Morning plasma free cortisol: inability to distinguish patients with mild Cushing syndrome from patients with pseudo-Cushing states. *Journal of Endocrinology Investigation*. 18: 696 –701.
8. Gallagher, P., Leitch, M. M, Massey, A. E, McAllister, Williams R. H, Young, A. H. Assessing cortisol and dehydro-epiandrosterone (DHEA) in saliva: effects of collection method. Journal of Psychopharmacol 2006; 20: 643-9.
9. Glass, A.R, Zavadil, III AP, Halberg F, Cornelissen G, Schaaf M. 1984 Circadian rhythm of serum cortisol in Cushing's disease. *Journal of Clinical Endocrinology and Metabolism*. 59:161–165.
10. Gold, P.W, Drevets, WC, Charney DS. New insights into the role of cortisol and the glucocorticoid receptor in severe depression. Biol Psychiatry 2002; 52: 381-5.
11. Ito, N., Ito, T., Kromminga A, et al. Human hair follicles display a functional equivalent of the hypothalamic-pituitary-adrenal axis and synthesize cortisol. FASEB J 2005; 19: 1332-4.
12. Jones, R. L., Owen, L.J., Adaway, J. E., Keevil, B. G. (2012). Simultaneous analysis of cortisol and cortisone in saliva using XLC-MS/MS for fully automated online solid phase extraction. *Journal of Chromatography*. 881-882:42-8.
13. Keevil Brian G., (2016). LC-MS/MS Analysis of steroids in the clinical laboratory, *Clinical Biochemistry*. doi: 10.1016/ 2016.04.009.
14. Lavker RM, Sun TT, Oshima H, et al. Hair follicle stem cells. *Journal of Investigative Dermatology Symposium Proceedings* 2003; 8: 28-38.
15. Loussouarn, G. African hair growth parameters. *British Journal of Dermatology* 2001; 145: 294-297.
16. Perogamvros, I., Owen, L.J., Keevil, B. G., Brabant, G., Trainer, P. J. (2010). Measurement of salivary cortisol with liquid chromatography-tandem mass spectrometry in patients undergoing dynamic endocrine testing. *Clinical Endocrinology* (Oxford) 72:17-2145.
17. Pichini, S., Altieri, I., Pellegrini, M., Pacifici R, Zuccaro P. Hair analysis for nicotine and cotinine: evaluation of extraction procedures, hair treatments, and development of reference material. Forensic Science International. 1997; 84: 243-52.
18. Raff, H., Singh, R.J. (2012). Measurement of late night salivary cortisol and cortisone by LC-MS/MS to assess preanalytical sample contamination with topical hydrocortisone. *Clinical Chemistry*. 58:947-948.
19. Raul, J. S, Cirimele, V., Ludes, B, Kintz, P. Detection of physiological concentrations of cortisol and cortisone in human hair. Clin Biochem 2004; 37: 1105-11.
20. Shimatsu, A, Kato Y, Sakami H, Nakai Y, Imura H. (1988). Phase-shifted diurnal rhythm of cortisol secretion in a patient with Cushing's syndrome due to adrenocortical adenoma. *Endocrinology Japan*. 35:261–266.
21. Spiehler, V. Hair analysis by immunological methods from the beginning to 2000. Forensic Science International 2000; 107: 249-59.
22. Tanada, N., Kashimura S, Kageura M, Hara K. Utility of caffeine analysis for forensic hair discrimination. Nihon Hoigaku Zasshi 1998; 52: 233-237.
23. Tourniaire, J, Chalendar, D, and Rebattu, B.1986 The 24-h cortisol secretory pattern in Cushing's syndrome. Acta Endocrinol (Copenh). 112:230 – 237.
24. Tunn, S., Mollmann, H, Barth, J., Derendorf, H., Krieg, M. Simultaneous measurement of cortisol in serum and saliva after different forms of cortisol administration. Clin Chem 1992; 38: 1491-4.
25. Umeda, T., Hiramatsu, R., Iwaoka, T., Shimada, T., Miura, F, Sato T. (2015). Use of saliva for monitoring unbound free cortisol levels in serum. Clinical Chemistry 110: 245-53.

26. Wennig R. Potential problems with the interpretation of hair analysis results. Forensic Science International. (2000) 107: 5-12.

27. Yamada, J., Stevens, B., de Silva, N et al. Hair Cortisol as a Potential Biologic Marker of Chronic Stress in Hospitalized Neonates. Neonatology 2007; 92: 42-49.

28. Yegles, M., Marson, Y., Wennig, R. Influence of bleaching on stability of benzodiazepines in hair.

Forensic Science International. 2000; 107: 87-92.

29. Yoon, J. B, Stephanie, S., Annette, M. K, Sonia, J., Andreas, H., Wieland, K., Uta, C., Alexander, G., Michael, S., Kai von K., Joachim, T. (2015). The hyporeactivity of salivary cortisol at stress test (TSST-C) in children with internalizing or externalizing disorders is contrastively associated with amylase. *Journal of Psychiatric Research 71*.78e8.

12/13/2020